Changes in Gene and Protein Expression and Reproduction in Grass Shrimp, *Palaemonetes pugio*, Exposed to Chronic Hypoxia.

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Aquatic Research Consortium (ARC)
National Oceanic & Atmospheric Administration
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ARC Objectives: Determine Molecular and Organismal Indicators of Effects of Natural and Anthropogenic Stressors in Estuarine Species

Grass Shrimp

Sheepshead Minnows

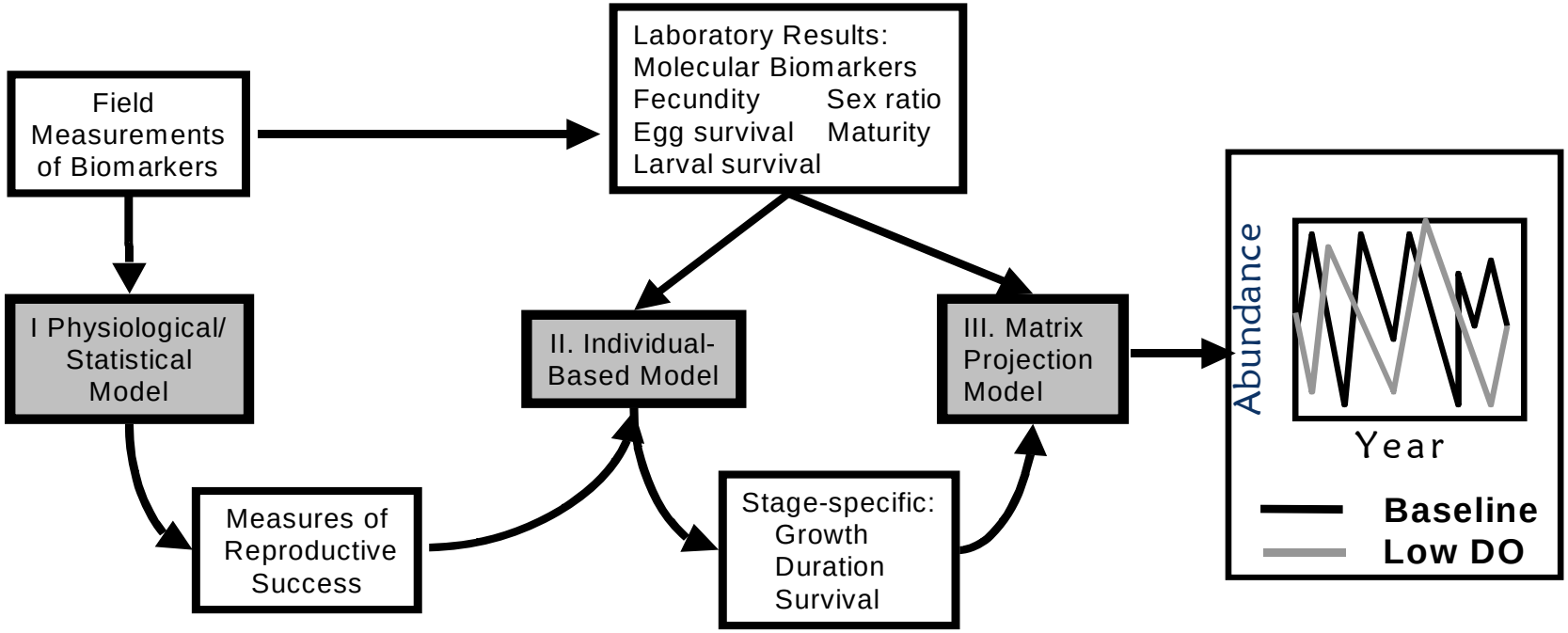
Molecular *Indicators: Gene Transcription Profiles and Protein Expression Profiles*

Better Understanding of Thermodynamics and Kinetics of Gene Array Hybridization (Alex Pozhitkov)

Organismal *(Ecologically Relevant) Indicators: Reproduction needed for scaling to the population level*

Conceptual model for scaling individual-level effects (biomarkers) of environmental stressors to the population level

with Dr. Kenny Rose - LSU

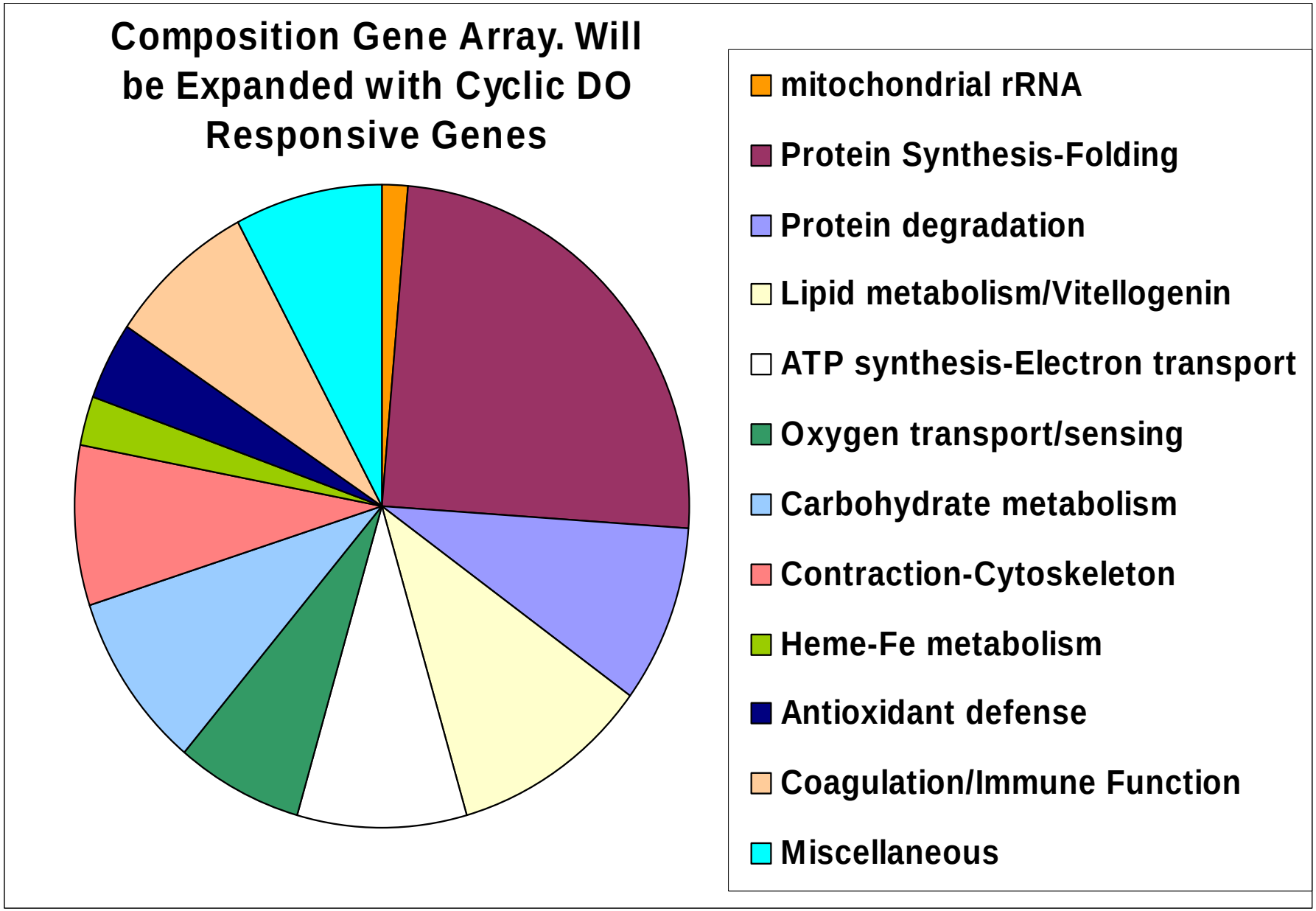


Aquatic Research Consortium – USM Environmental Stressors

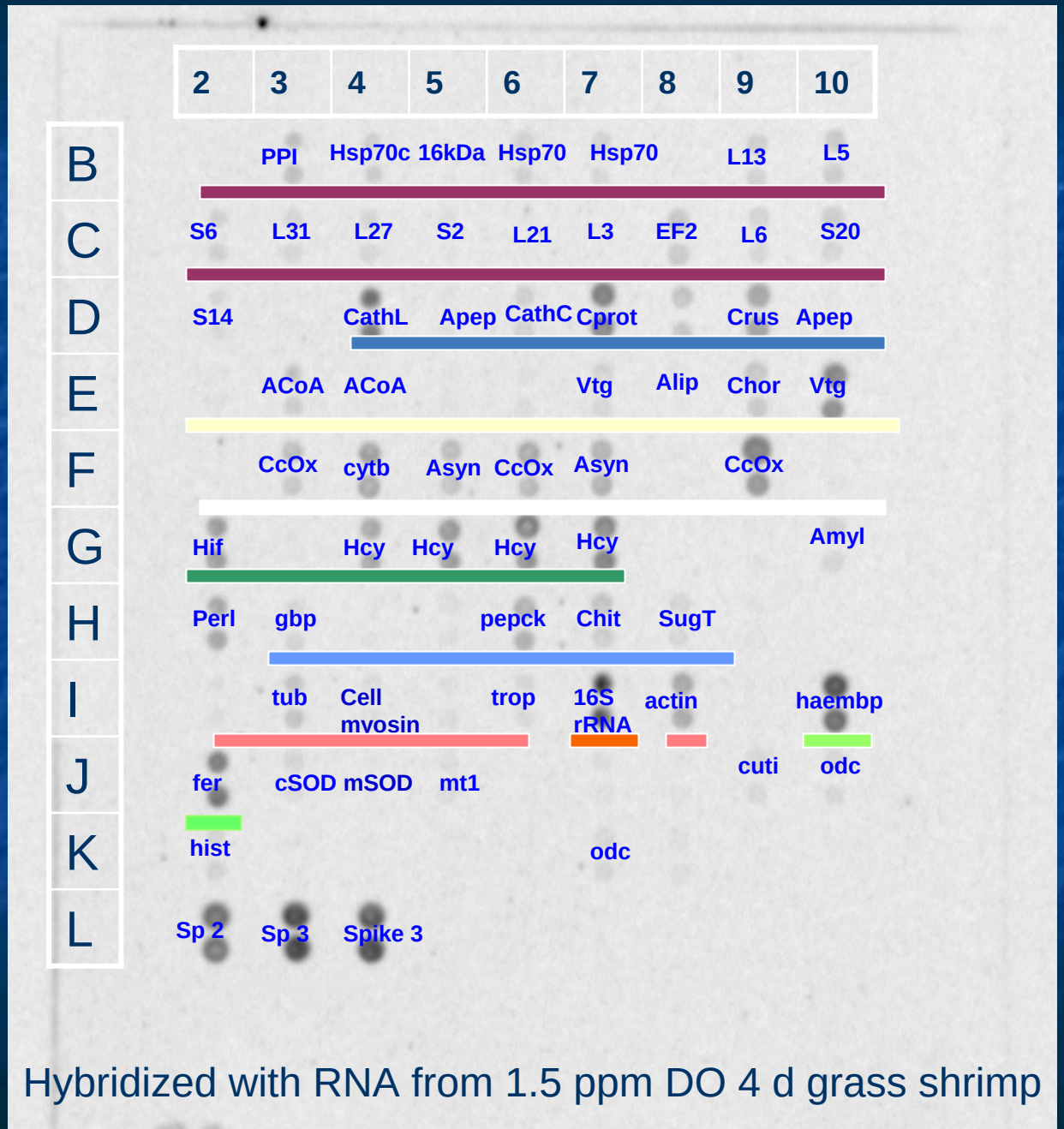
- Chronic hypoxia – stratified systems
- Intermittent hypoxia – tidal creeks
- Polycyclic Aromatic Hydrocarbons – PAHs
- Endocrine disruptors (EDCs)
- Toxic metals (chromium and cadmium)
- Interaction between hypoxia and PAHs, EDCs and metals

Objectives Grass Shrimp Hypoxia Project

- Clone hypoxia (chronic and intermittent)-responsive genes using subtractive hybridization and use clones to construct macroarrays
- Use the gene arrays to identify hypoxia-responsive genes in grass shrimp in controlled laboratory experiments
- Identify hypoxia-responsive reproductive parameters in laboratory experiments – needed to scale up to the population level
- Evaluate laboratory-derived parameters in field-collected grass shrimp

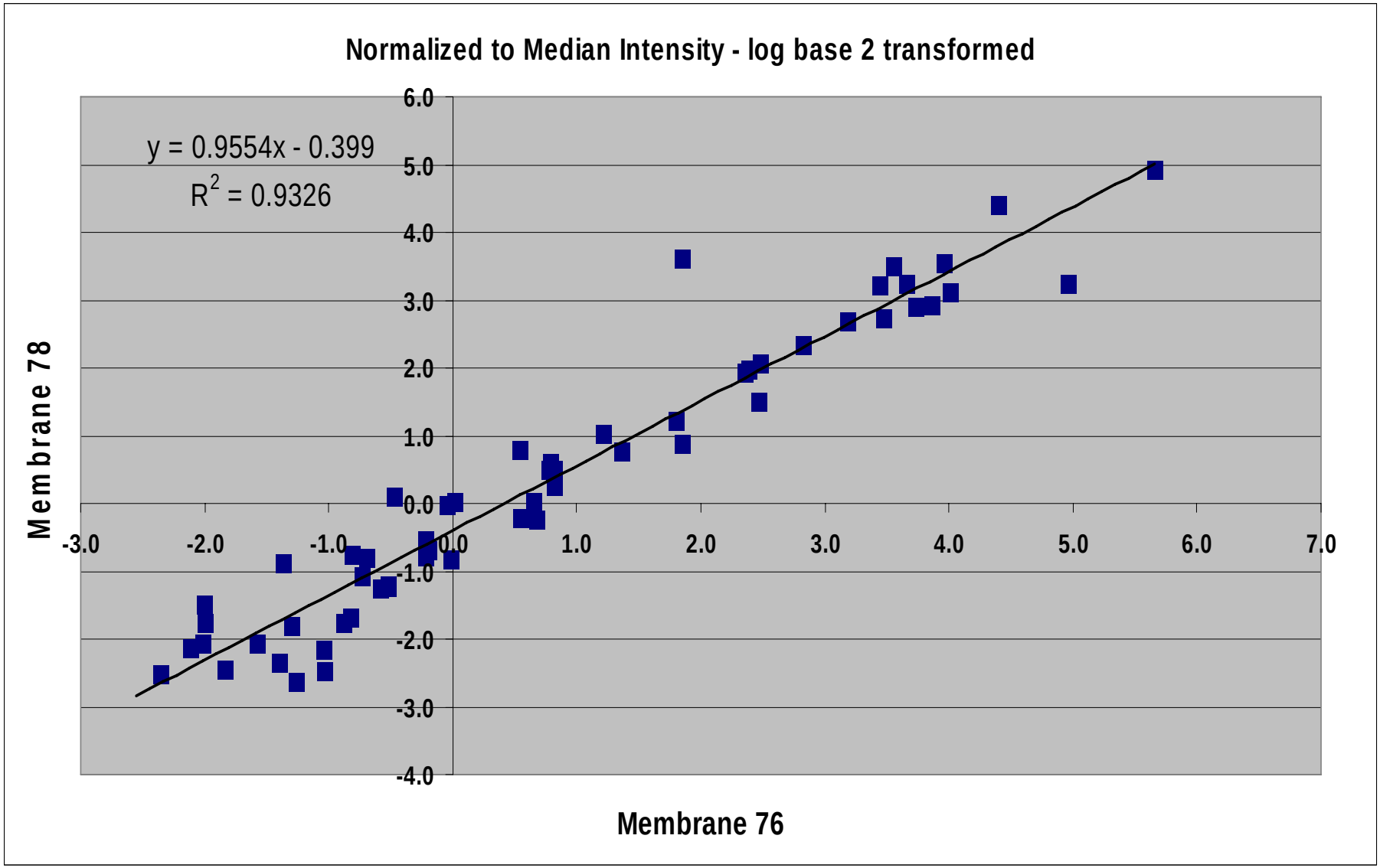

Composition Gene Array. Will be Expanded with Cyclic DO Responsive Genes
mitochondrial rRNA
Protein Synthesis-Folding
Protein degradation
Lipid metabolism/Vitellogenin
ATP synthesis-Electron transport
Oxygen transport/sensing
Carbohydrate metabolism
Contraction-Cytoskeleton
Heme-Fe metabolism
Antioxidant defense
Coagulation/Immune Function
Miscellaneous

Macroarray Membrane Layout

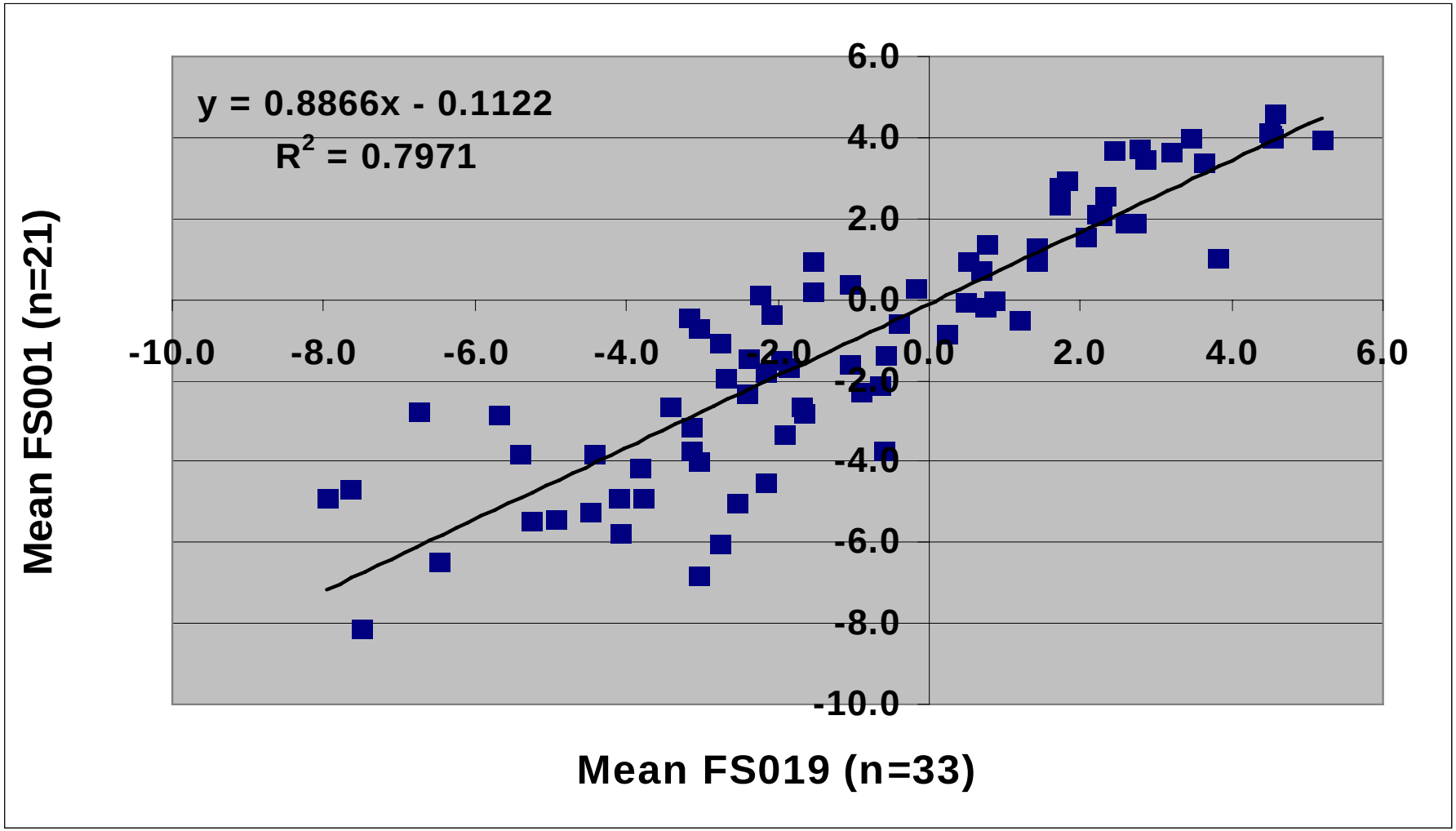


Hybridized with RNA from 1.5 ppm DO 4 d grass shrimp

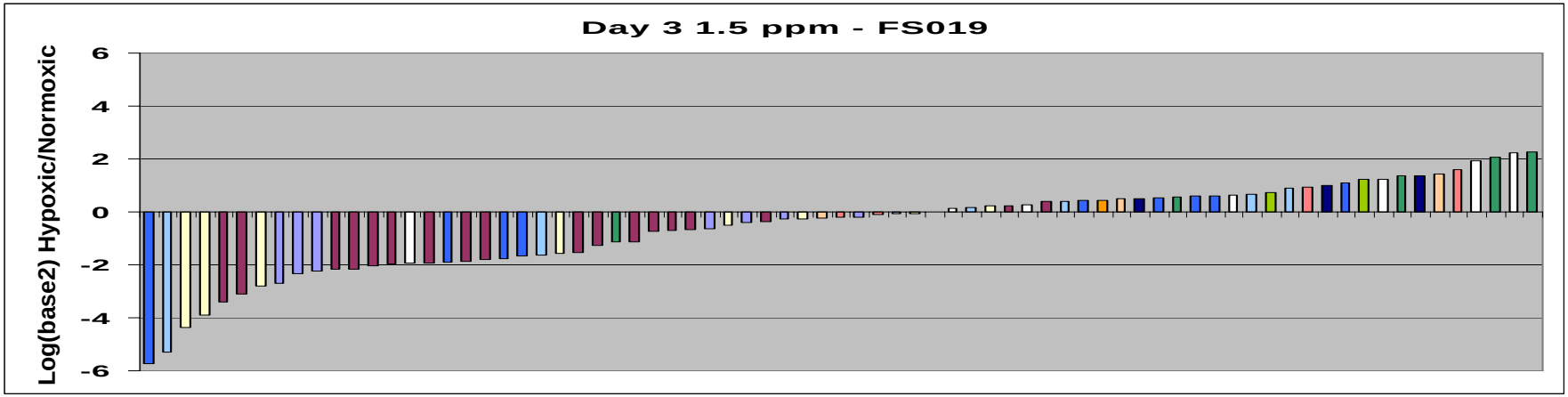
Variability Due to Experimental Error is Small

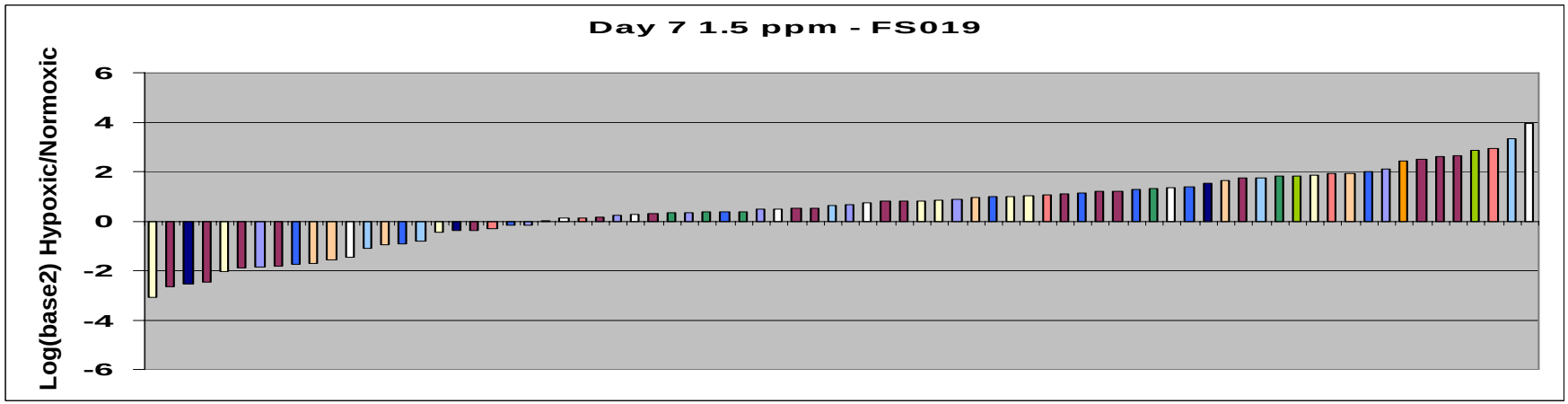


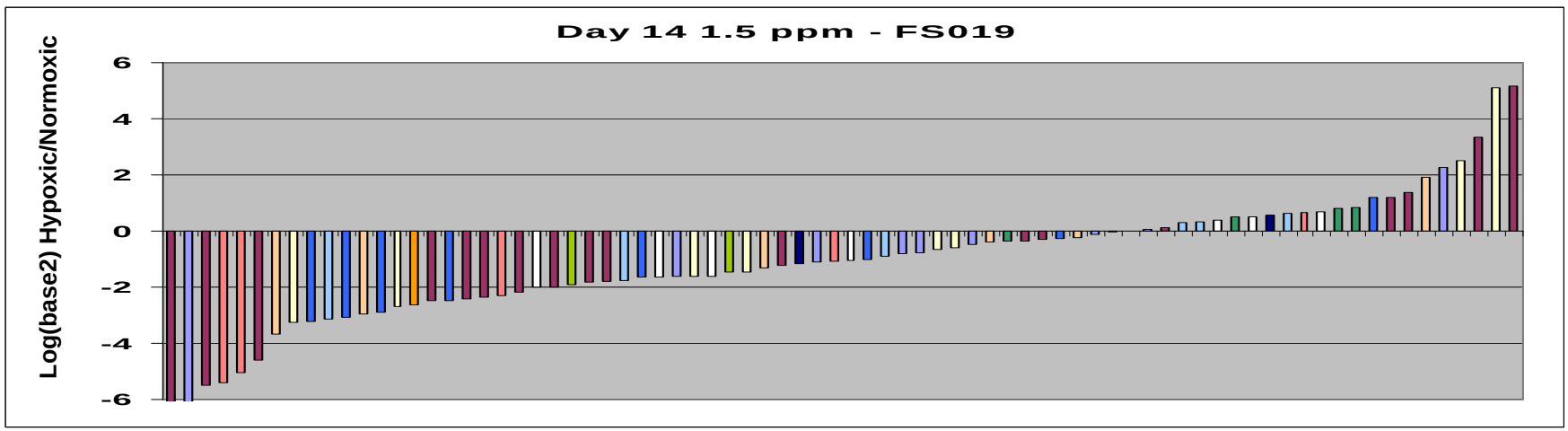
Comparison Mean Normalized Gene Intensities of Normoxic Control Shrimp from Two Experiments

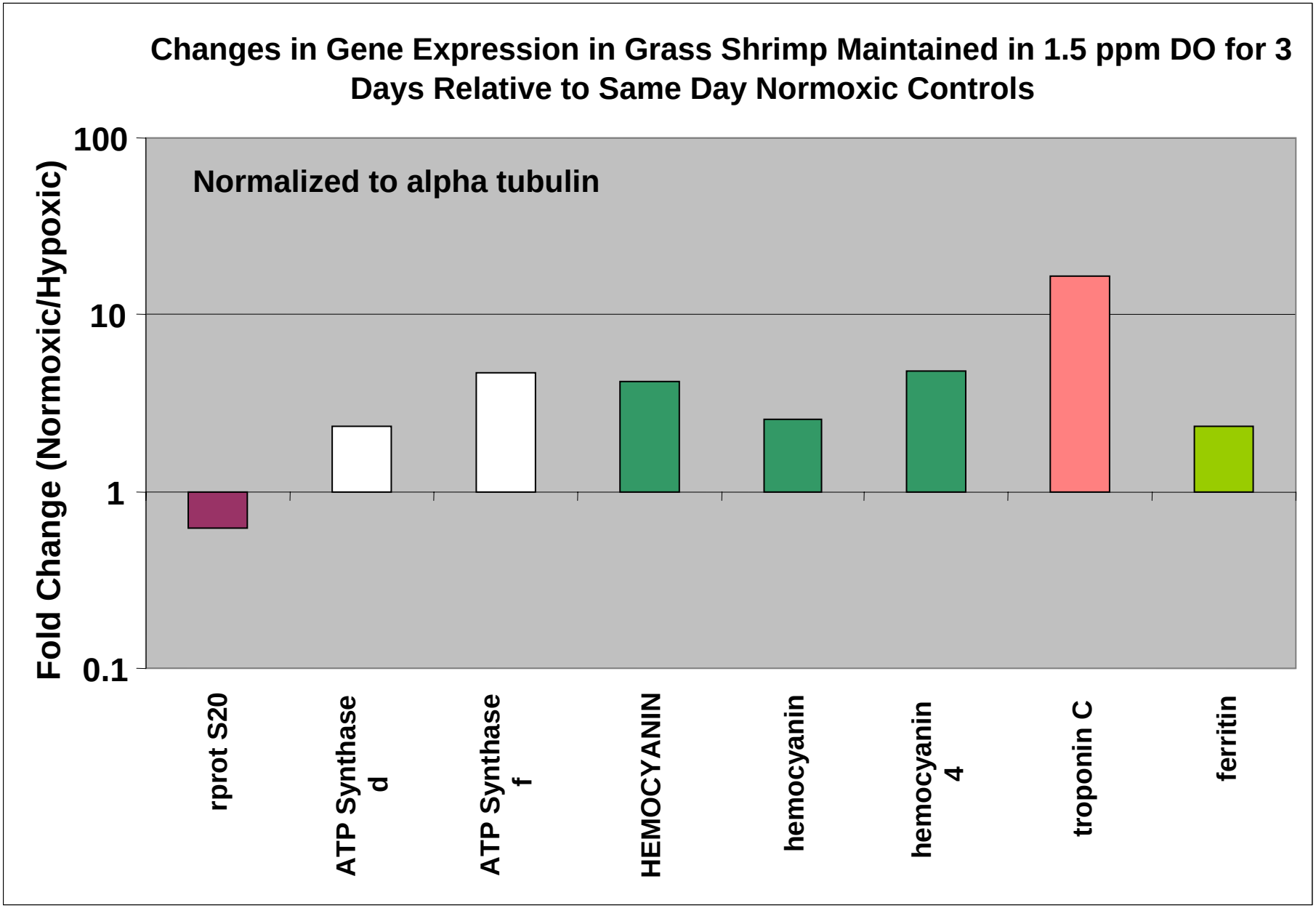


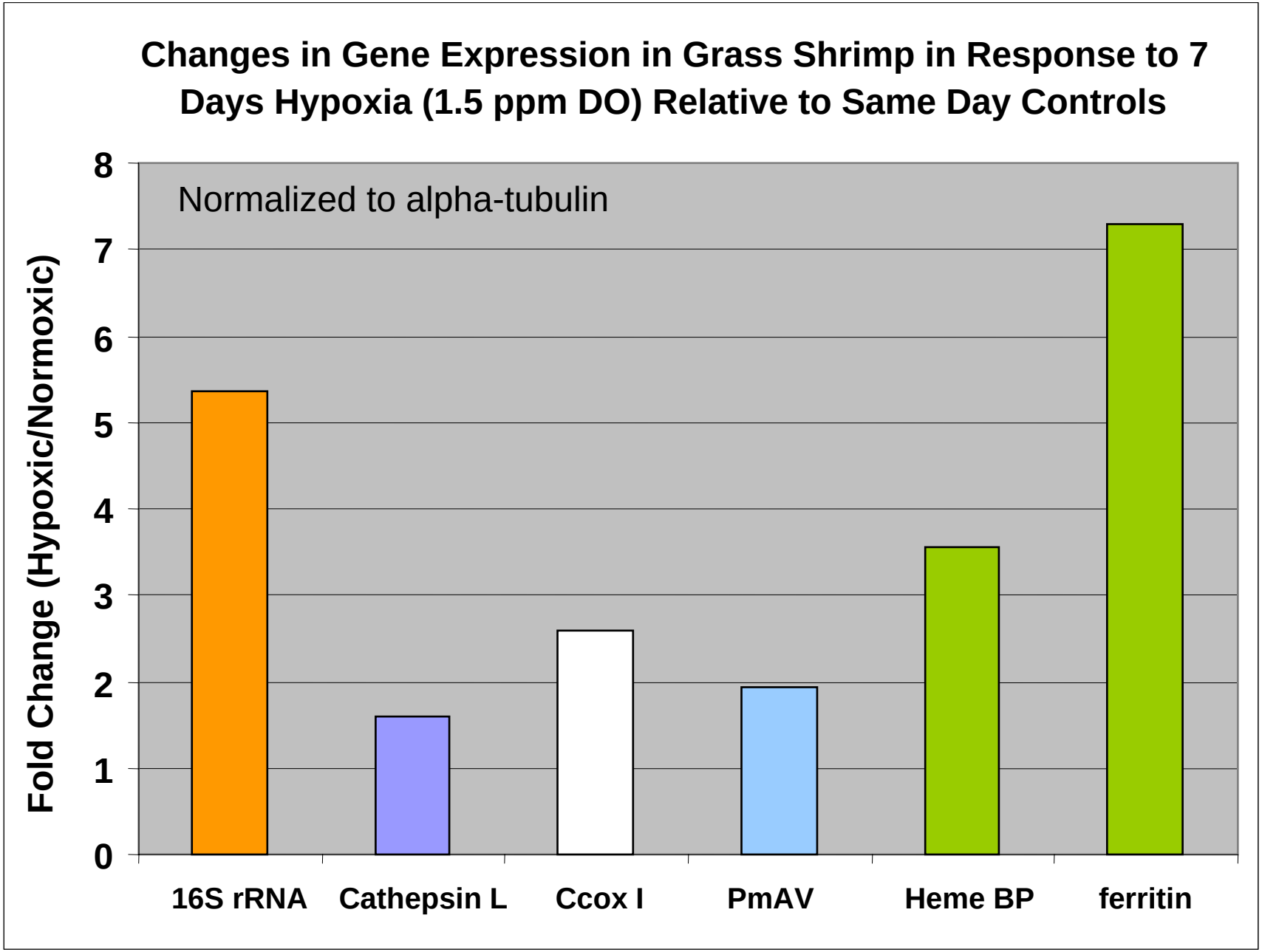
Variability of Gene Expression Levels Among Shrimp is Modest

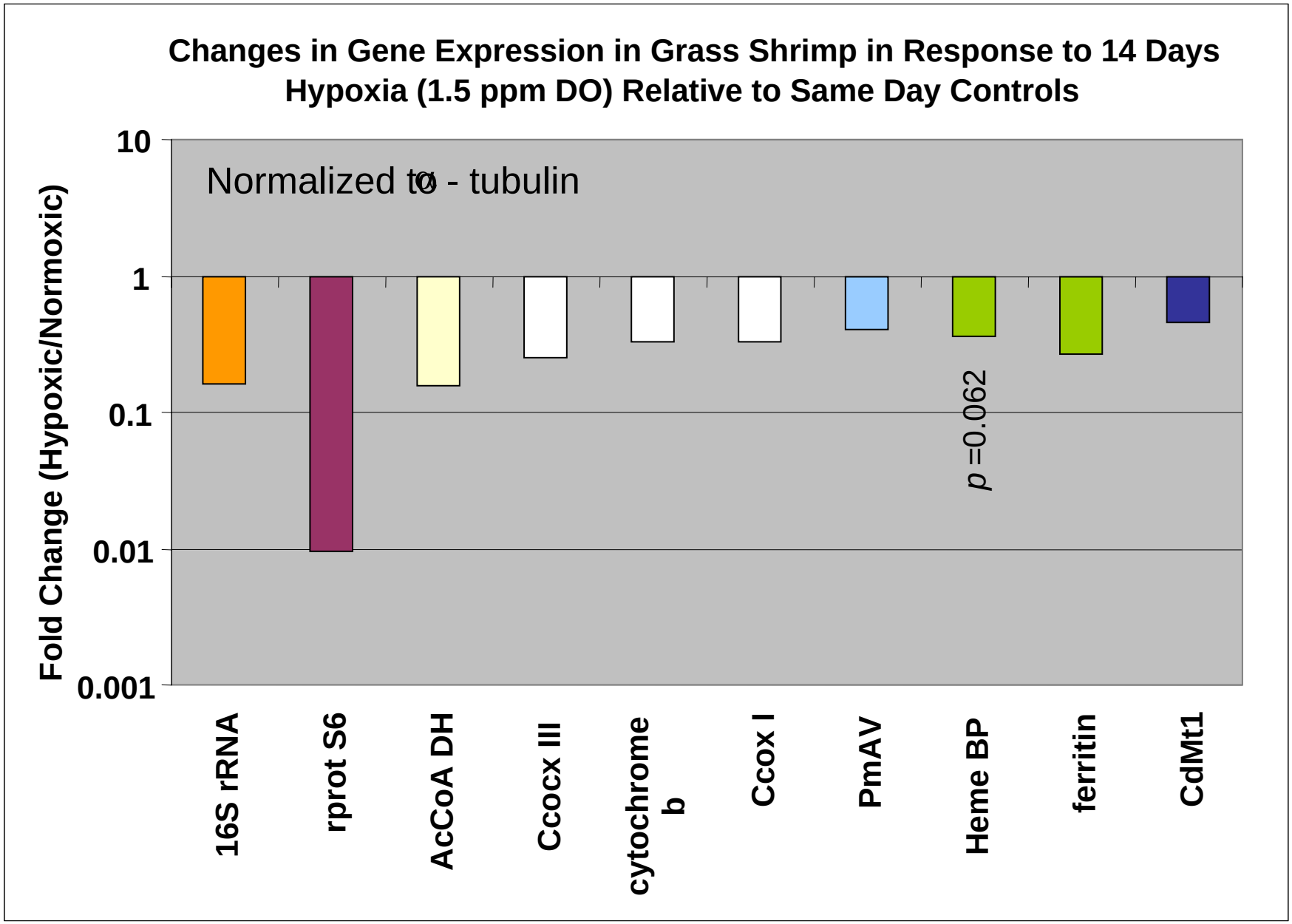

Day 3 1.5 ppm - FS019
Log(base2) Hypoxic/Normoxic
6
4
2
0
-2
-4
-6


Day 7 1.5 ppm - FS019
Log(base2) Hypoxic/Normoxic
6
4
2
0
-2
-4
-6


Day 14 1.5 ppm - FS019
Log(base2) Hypoxic/Normoxic
6
4
2
0
-2
-4
-6


Changes in Gene Expression in Grass Shrimp Maintained in 1.5 ppm DO for 3 Days Relative to Same Day Normoxic Controls
Normalized to alpha tubulin
Fold Change (Normoxic/Hypoxic)
100
10
1
0.1
rprot S20
ATP Synthase d
ATP Synthase f
HEMOCYANIN
hemocyanin
hemocyanin 4
troponin C
ferritin


Changes in Gene Expression in Grass Shrimp in Response to 7 Days Hypoxia (1.5 ppm DO) Relative to Same Day Controls
Normalized to alpha-tubulin
Fold Change (Hypoxic/Normoxic)
0
1
2
3
4
5
6
7
8
16S rRNA
Cathepsin L
Ccox I
PmAV
Heme BP
ferritin


Changes in Gene Expression in Grass Shrimp in Response to 14 Days Hypoxia (1.5 ppm DO) Relative to Same Day Controls
Normalized to α - tubulin
Fold Change (Hypoxic/Normoxic)
10
1
0.1
0.01
0.001
p =0.062
16S rRNA
rprot S6
AcCoA DH
Ccocx III
cytochrome b
Ccox I
PmAV
Heme BP
ferritin
CdMt1

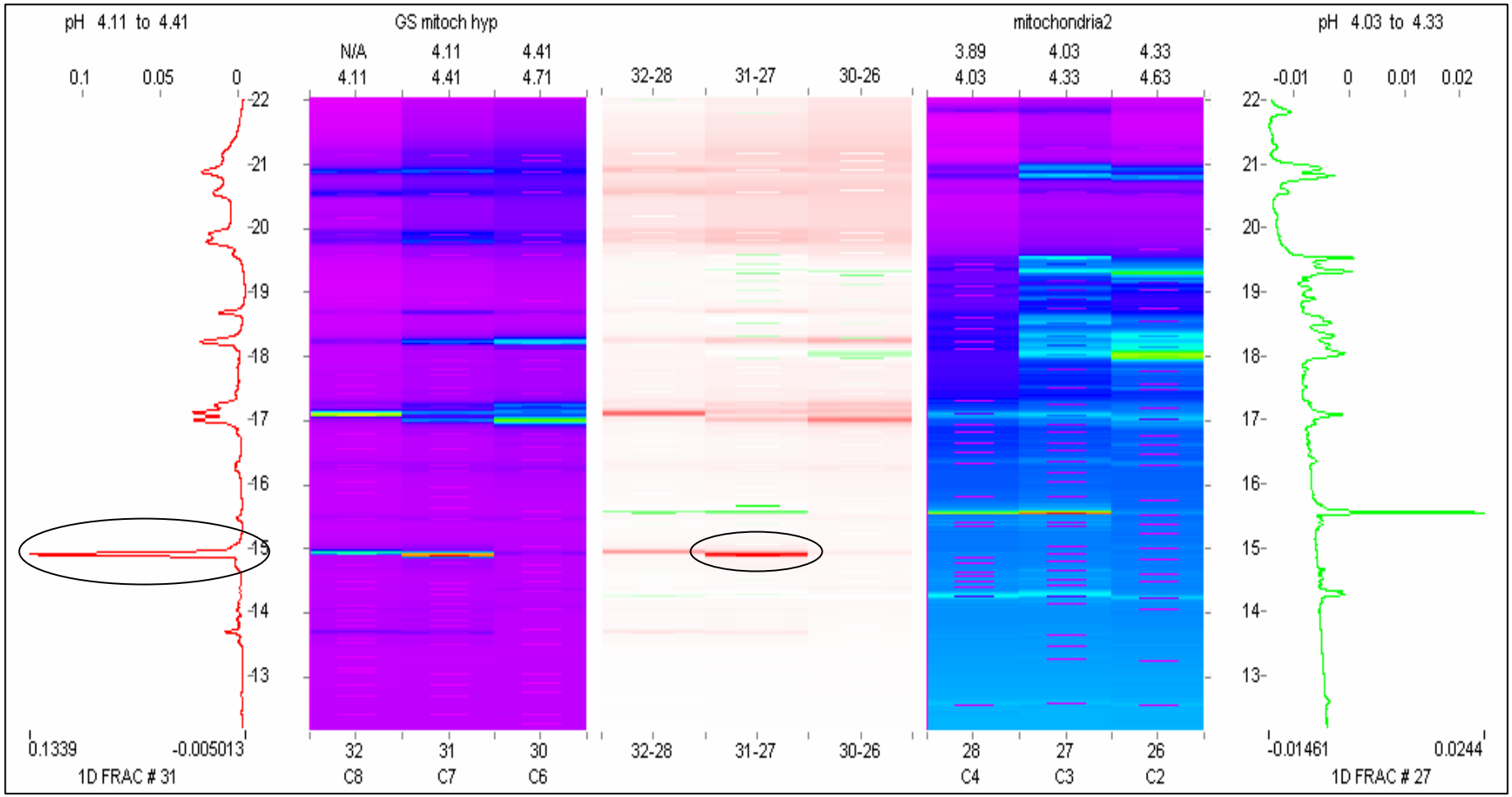
Conclusion

1. Mitochondrial genome-encoded 16S rRNA, cytochrome b and cytochrome c oxidase subunits I and III
2. Nuclear-encoded mitochondrial proteins (ATP synthase subunits) and
3. Oxygen transport proteins (Hemocyanin subunits) appear to be robust molecular indicators of chronic hypoxia (1.5 ppm DO) exposure

Conclusion- continued

- ✓ Changes in transcriptional profiles of these genes also provide information on the duration of exposure
- ✓ Real Time q-PCR confirms results of gene arrays (9 out of 10 genes tested)

Muscle Mitochondrial Proteins Separated by IEF Followed by Reverse Phase Chromatography



Hypoxic 12 Days *Difference* *Normoxic*

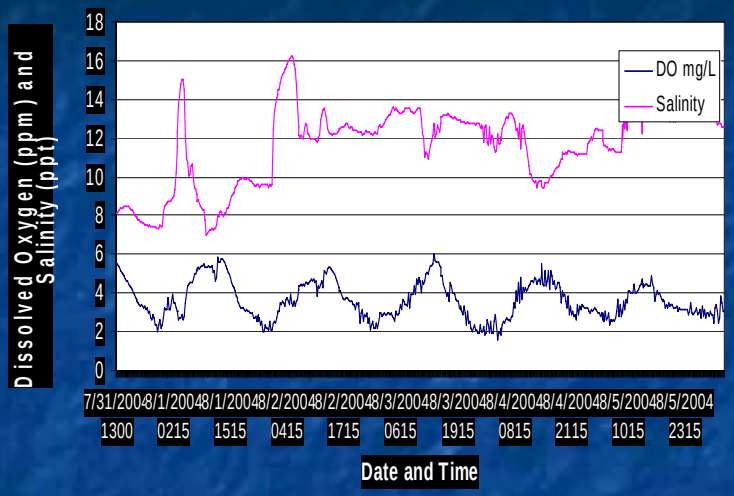
Proteins Differentially Expressed in Fraction #31 from Hypoxic Mitochondria Determined by MALDI MS and LC/MS/MS

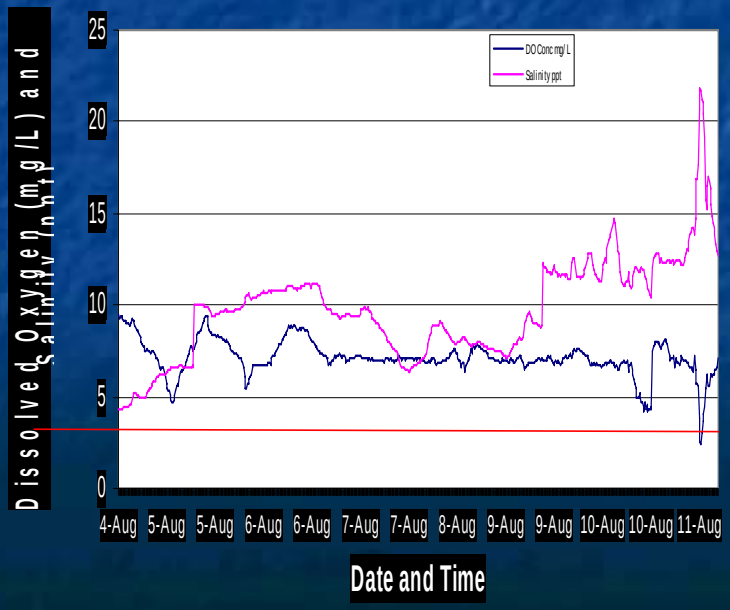
- NADH Dehydrogenase subunit 2 – encoded by mitochondrial genome –electron transport – contains iron-sulfur (cysteine) clusters
- Aconitase – TCA cycle enzyme – contains iron-sulfur (cysteine) clusters
- Mitochondrial large ribosomal subunit L1
- Serine acetyltransferase – cysteine biosynthesis (both cytosolic and mitochondrial isoforms)

Gene expression profiles of grass shrimp form normoxic and hypoxic field sites

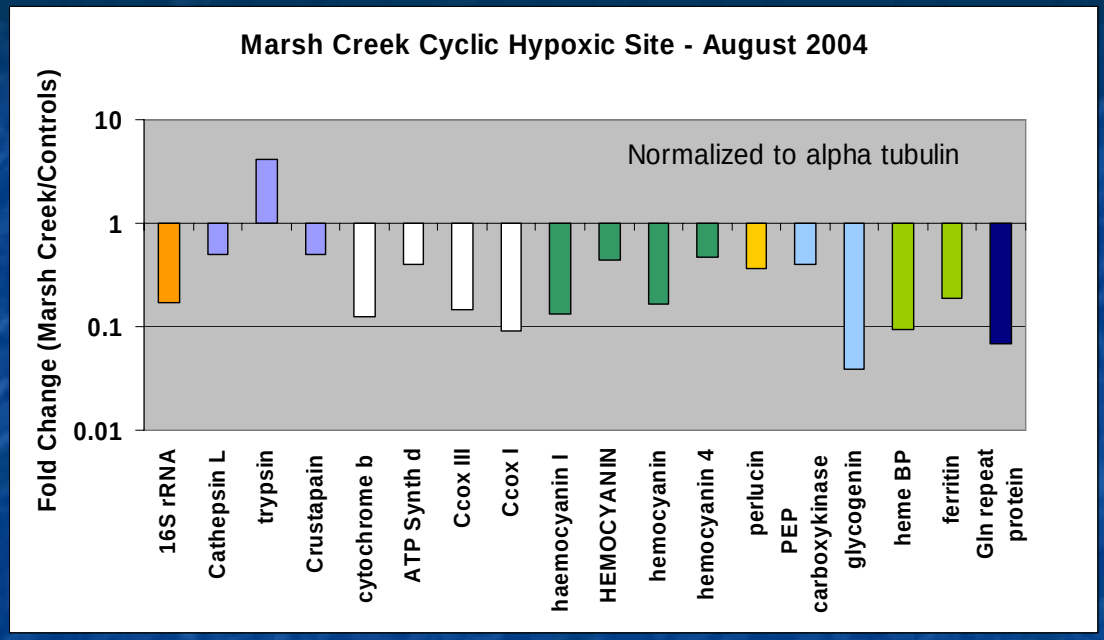
Pensacola Bay, August 2004

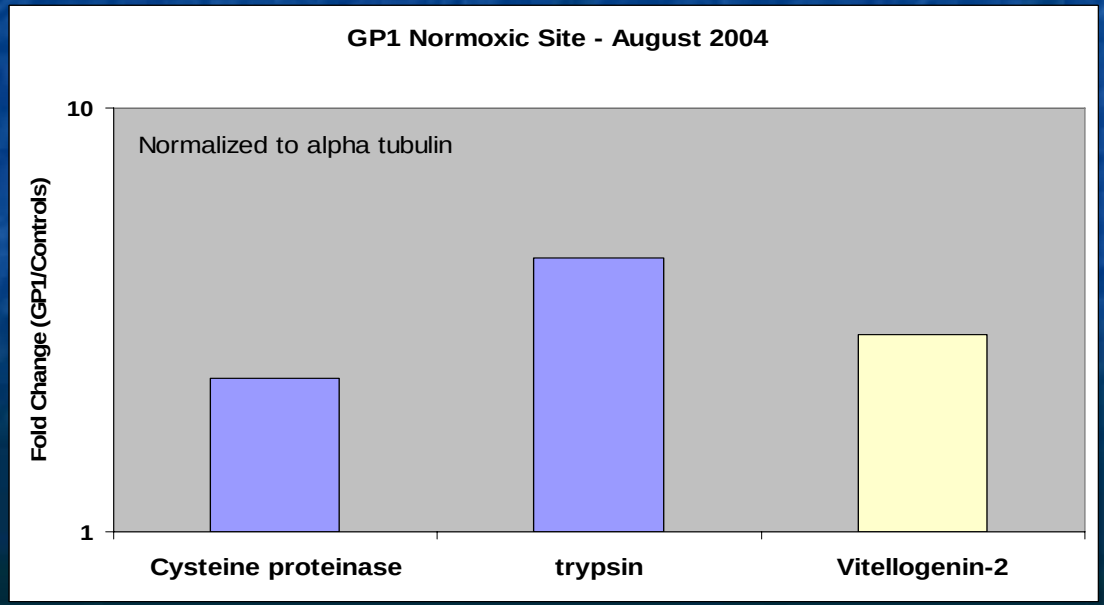
Water Quality



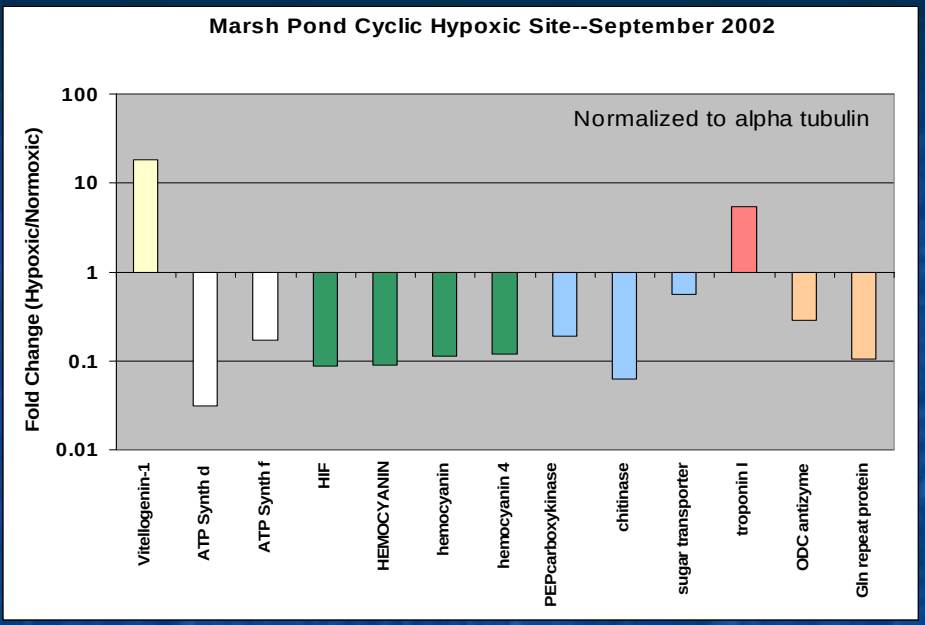


Gene Expression Relative to Normoxic Controls

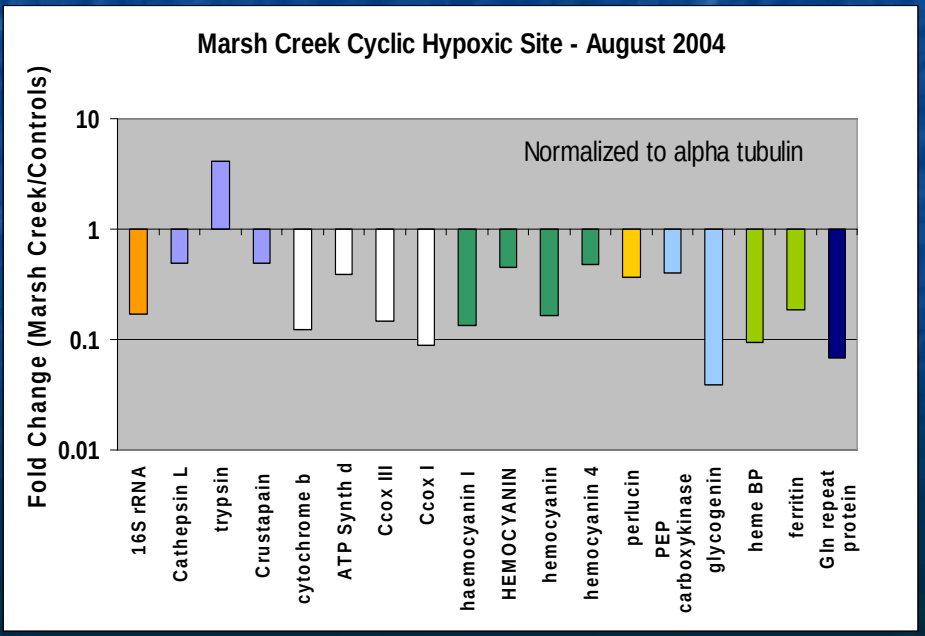


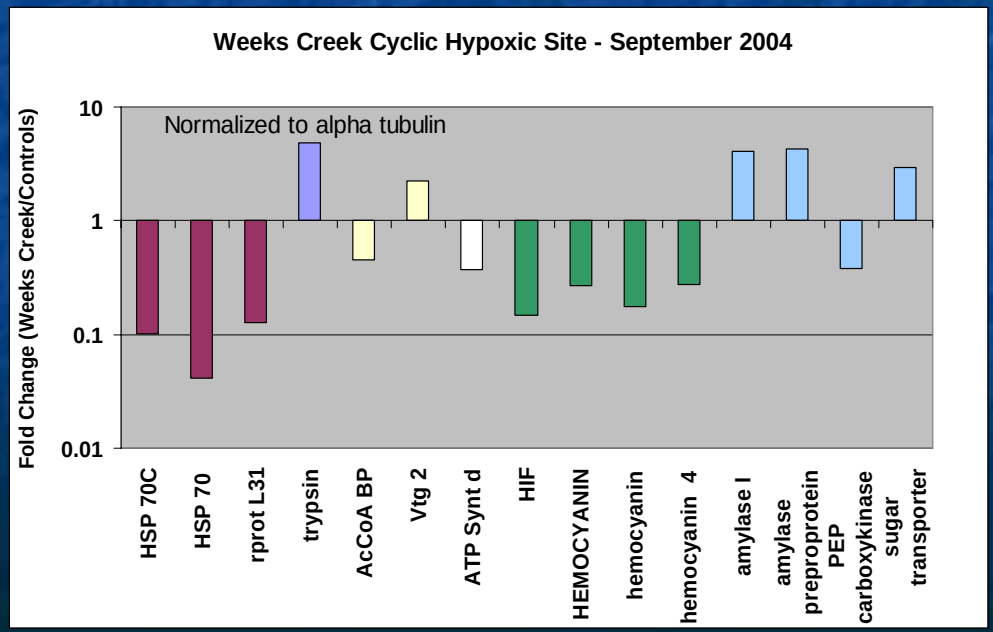


Gene Expression Relative to Normoxic Controls

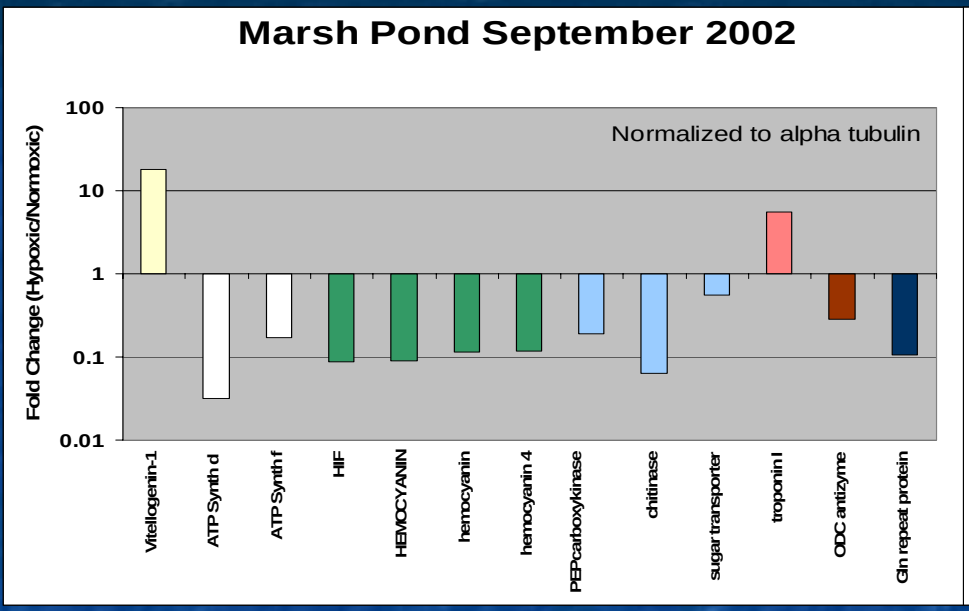


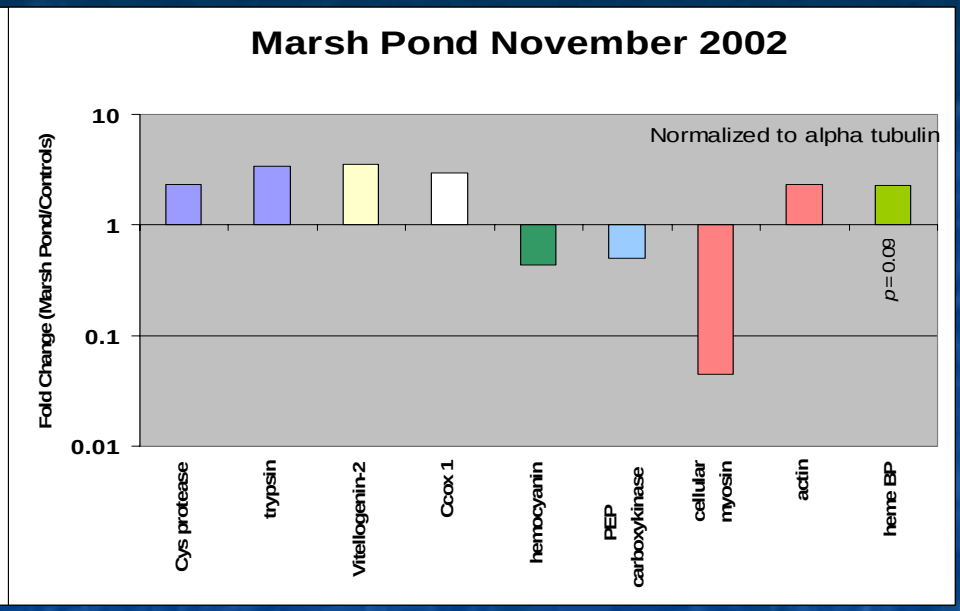
Similar across years and sites

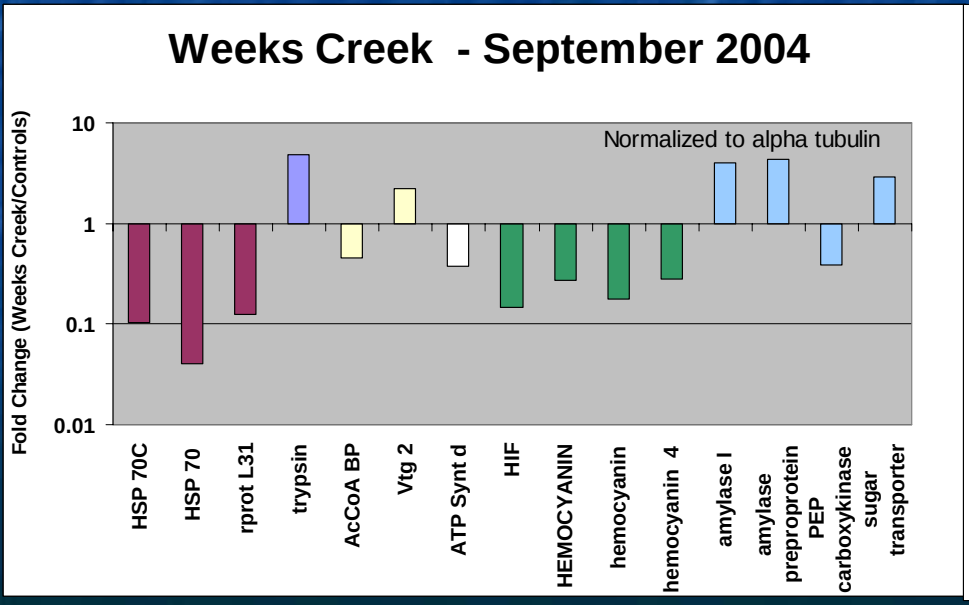


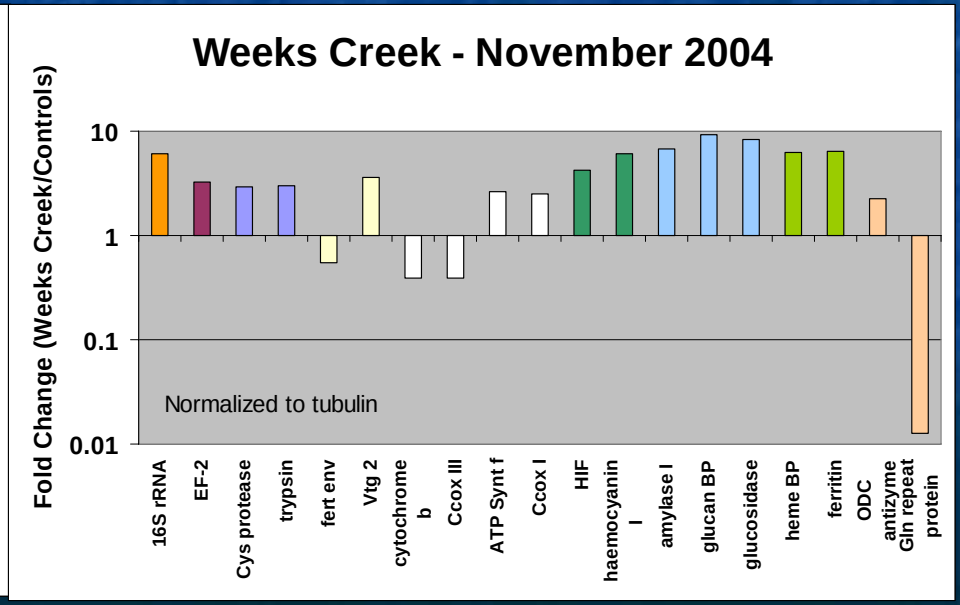


Seasonal Change/Recovery in Gene Expression









Summary Field Gene Expression

Grass shrimp from cyclic DO sites show distinct changes in gene expression profiles, not observed in shrimp from normoxic control sites

1. Downregulation of mitochondrial gene (cyt b and CcoxI and III) transcription similar to changes induced by chronic 1.5 ppm DO exposures in the lab

2. Downregulation of ATP synthase transcription in late summer (as observed after 7 days cyclic DO in the lab), hemocyanin (not observed in lab exposures), indicative of anaerobic metabolism

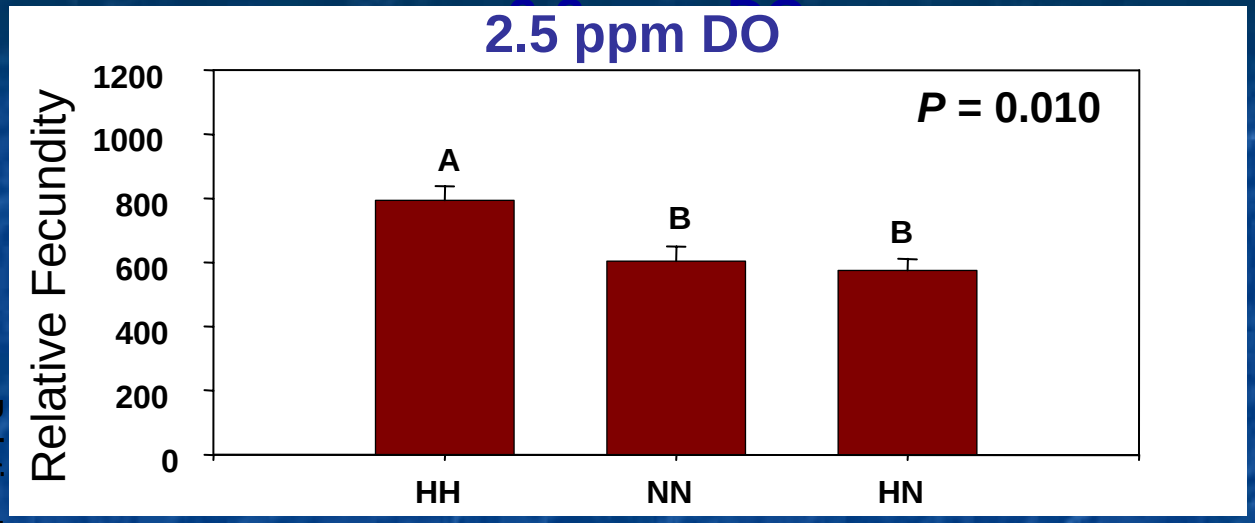
3. Return to aerobic, oxidative metabolism in late fall

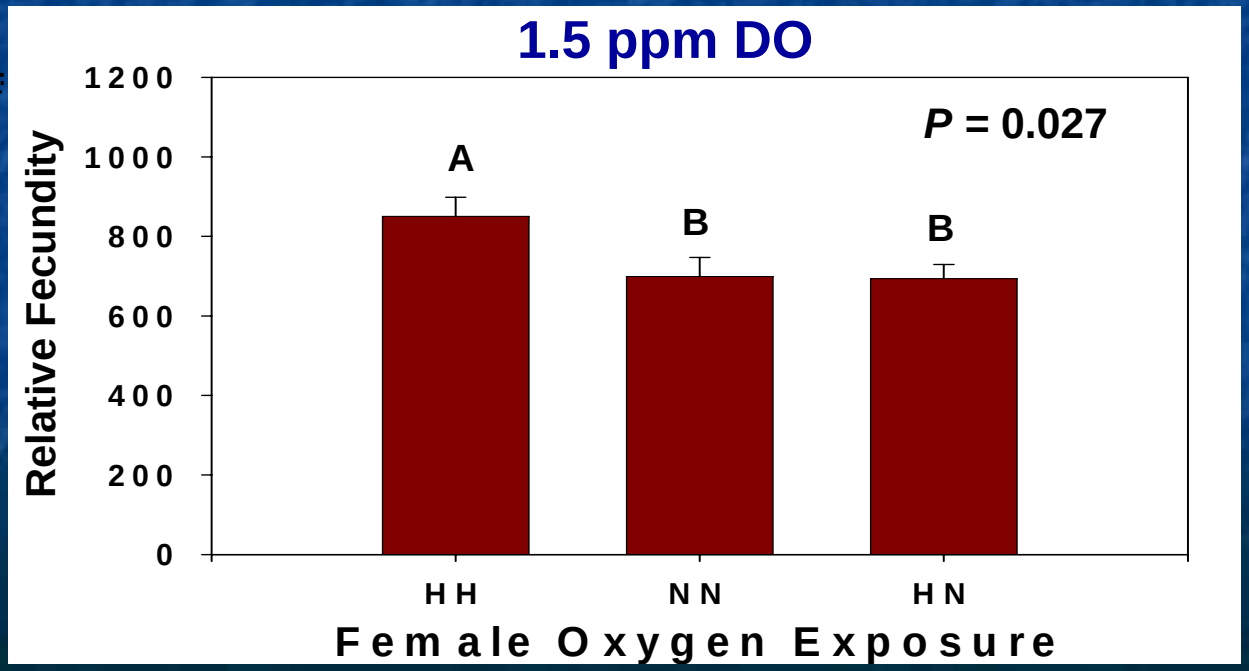
4. Expression patterns similar across sites and years

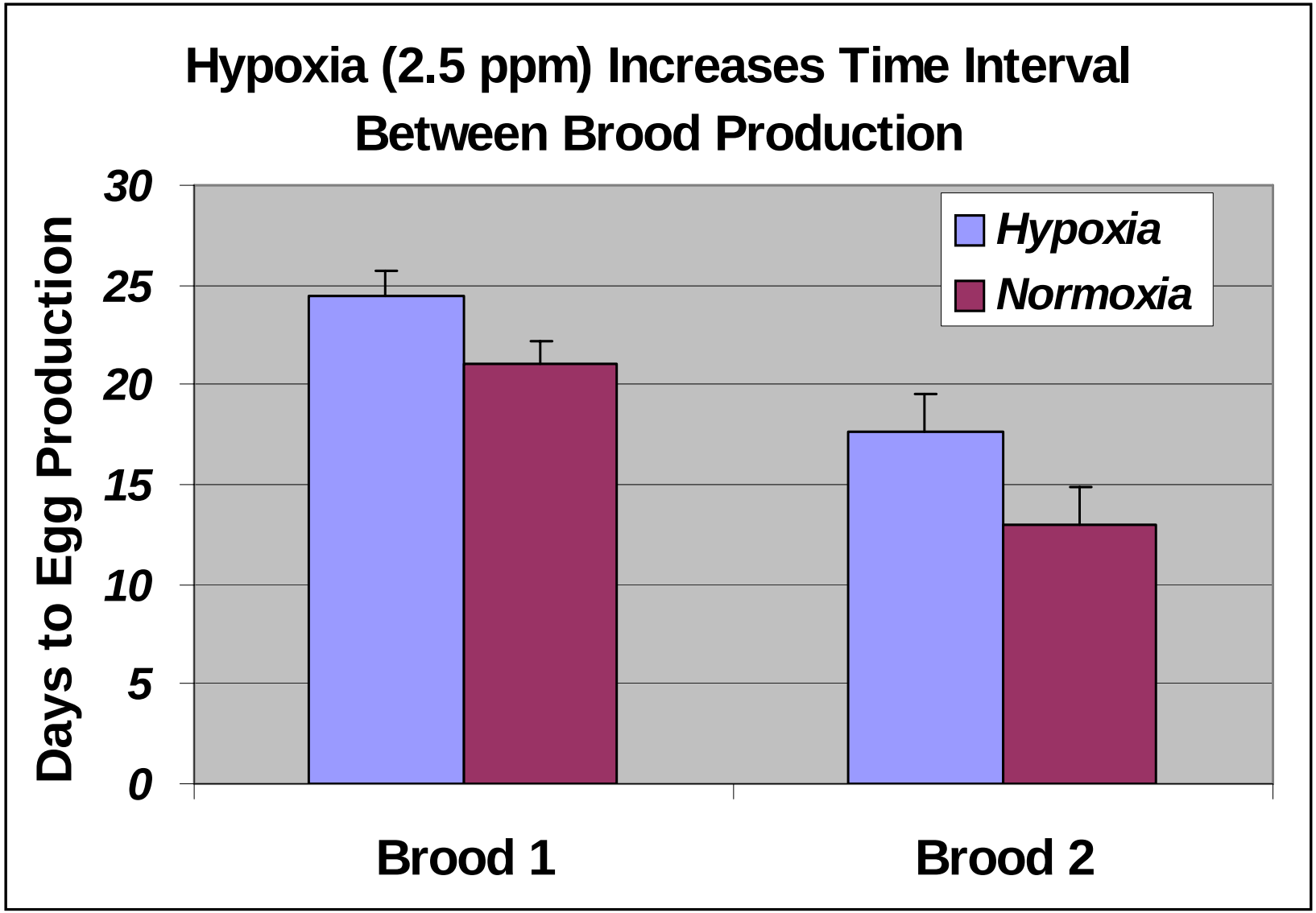
Reproductive Studies

- In the laboratory: chronic hypoxia, cyclic DO
- In the field

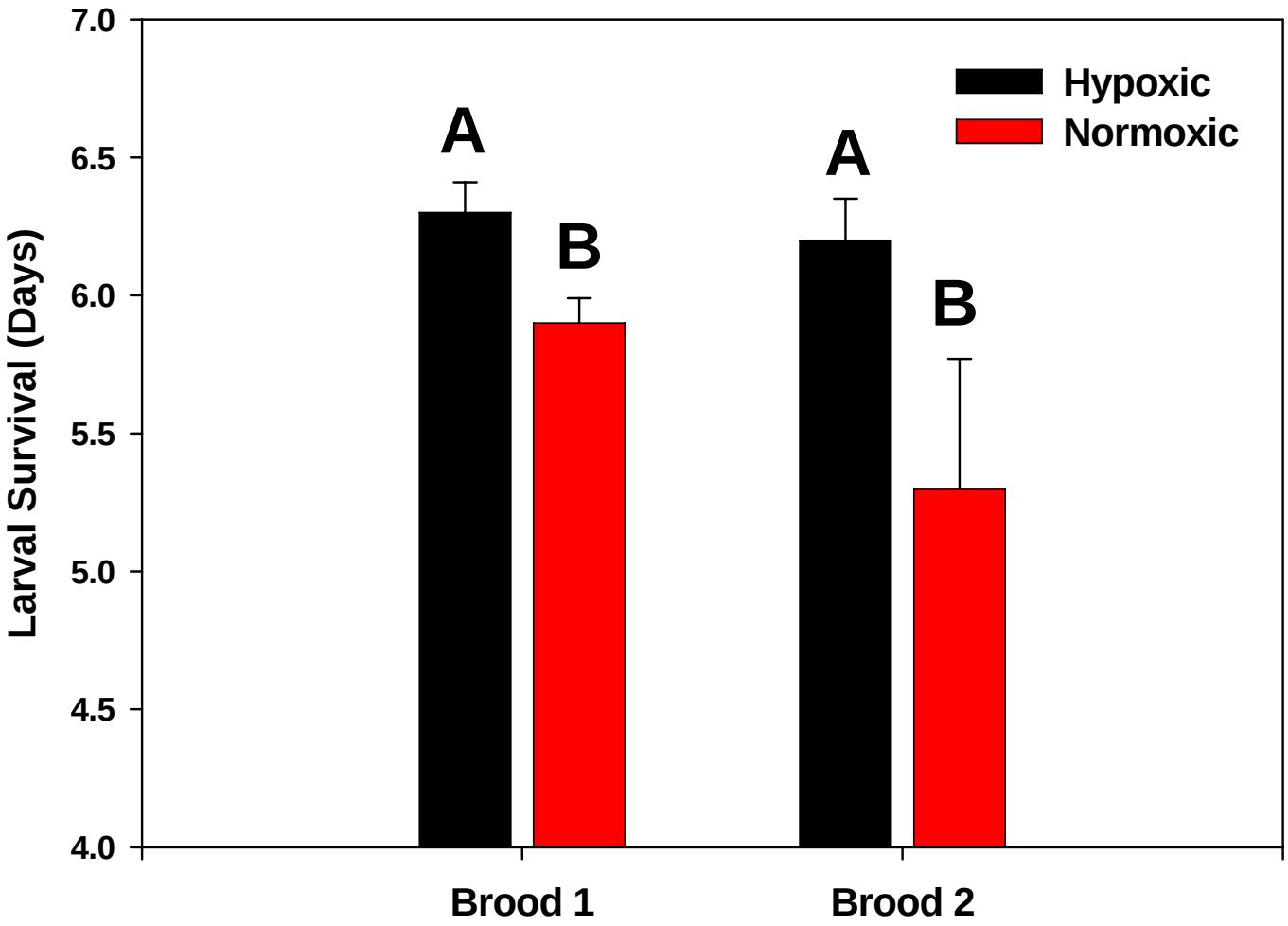
Chronic Hypoxia Increases Grass Shrimp Fecundity



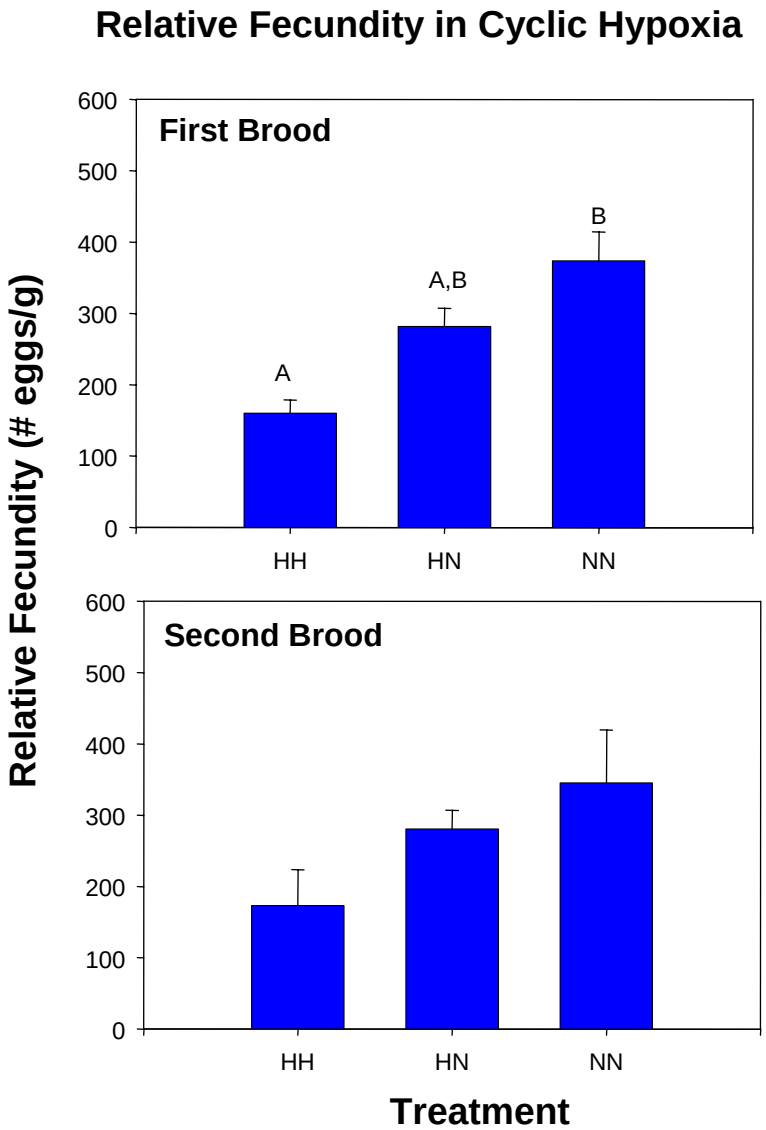


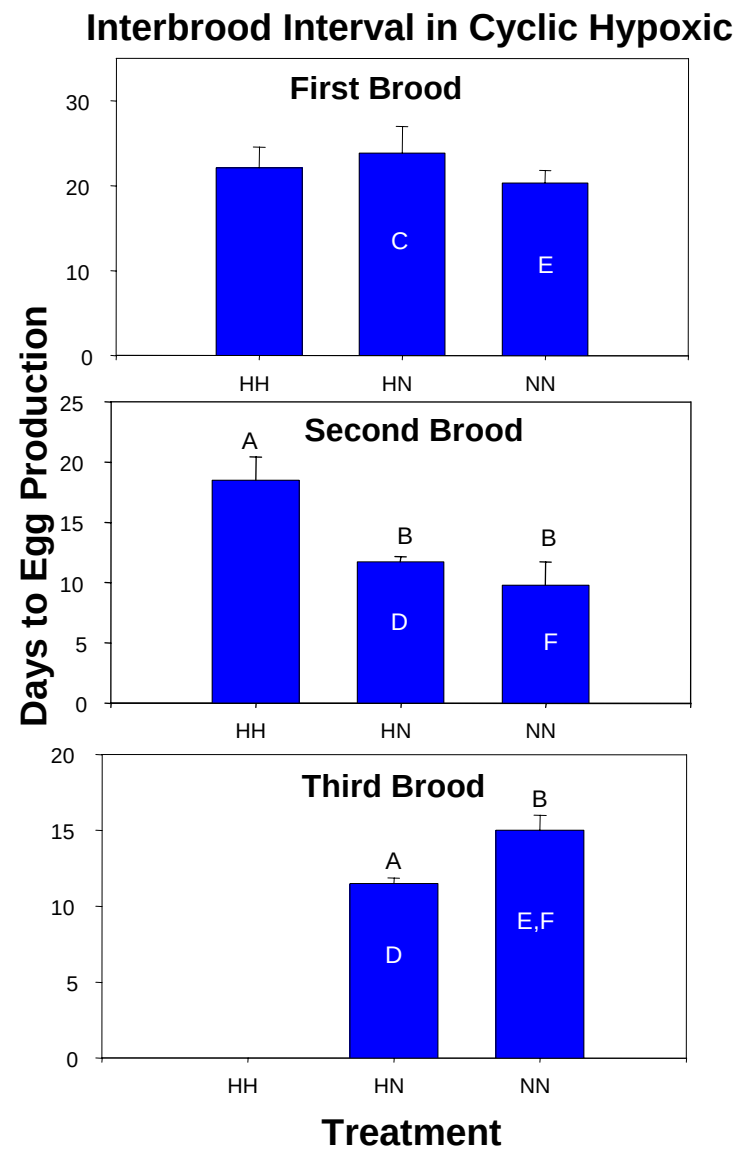

Hypoxia (2.5 ppm) Increases Time Interval Between Brood Production
Days to Egg Production
30
25
20
15
10
5
0
Hypoxia
Normoxia
Brood 1
Brood 2

Hypoxia (2.5 ppm DO) Increases Survival of Starved Larvae from Hypoxic Females



Effects of Cyclic Hypoxia on Grass Shrimp Reproduction Laboratory Experiments



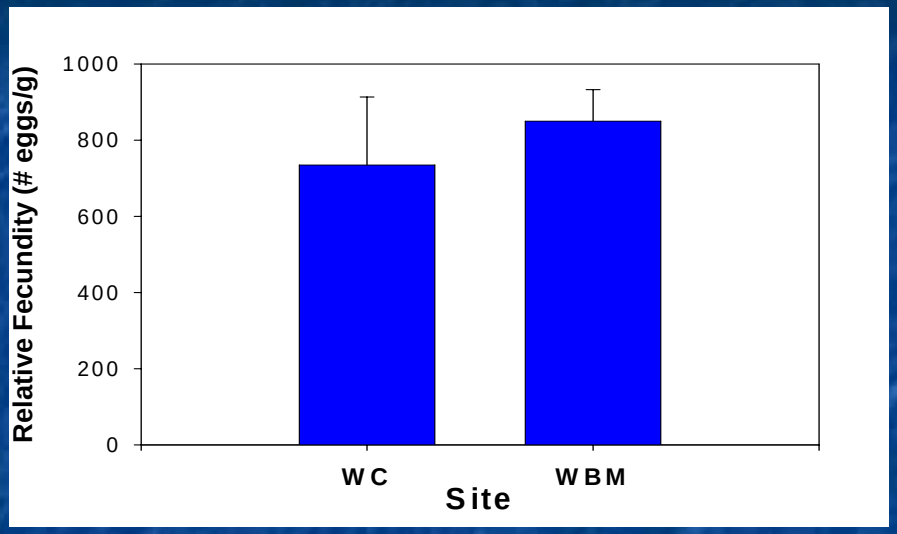


Conclusions

- ✓ Hypoxia induces time and dose-dependent changes in gene expression in grass shrimp
- ✓ Chronic hypoxia stimulates production of more and “healthier” eggs and increases time interval between broods
- ✓ Intermittent diurnal hypoxia decreases fecundity and increases time interval between broods

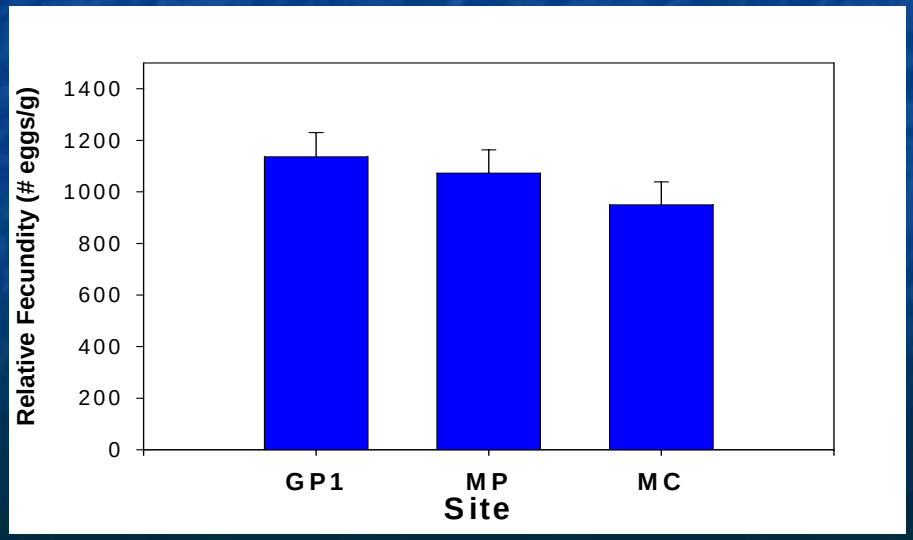
Reproduction of Grass Shrimp in the Field

Weeks Bay, July 2004



Station	% Egged	Ratio M:F
WC	18.2%	1:1
WBM	44.9%	1.3:1

Pensacola Bay, August 2004



Station	% Egged	Ratio M:F
GP1	75.8%	1.1:1
MC	45.8%	1.5:1
MP	29.5%	1.25:1

Conclusions

- There are distinct differences in gene expression profiles in grass shrimp from cyclic DO marsh sites and marsh edge, normoxic open bay sites
- There are distinct differences in reproductive function in grass shrimp from cyclic DO field sites and normoxic sites
- The two responses may be combined to provide diagnostic and predictive tools to asses effects of hypoxia on crustacea at the individual and population level

Future Directions-Cyclic DO SSH Gene Libraries

- ✓ We have cloned and sequenced many genes from a Cyclic DO SSH library. Several of these genes are not present in the chronic SSH DO library and hence may be specific for cyclic DO

- ✓ 8 Genes that are involved in sulfur redox metabolism and (possibly) in controlling cysteine and glutathione levels – a classic response to oxidative stress

Future Directions-Cyclic DO Gene Libraries

- ✓ Multiple copies of vitellogenin in downregulated library – may have reproductive consequences
- ✓ Construction of cyclic-DO sensitive gene chips is underway (~ 700 genes Tiandoa Li)
- ✓ Lab cyclic DO exposures have been completed
- ✓ Field studies in cyclic DO sites in September 2006 have been completed

Graduate Students

1. Laura Hendon (M.S): Interaction between chronic/ intermittent hypoxia and PAHs in sheepshead minnow embryos (ARC)
2. Natasha Sharp (Ph.D.): Blue crab hemocyte subtracted libraries. Mechanisms of control of antimicrobial peptide synthesis (ARC).
3. Tiandao Li (Ph.D): Grass Shrimp chronic/intermittent DO (CEER/ARC)
4. Art Karels (Ph.D.): Control of HIF Prolyl Hydroxylase by hypoxia and estrogens in sheepshead minnows (CEER/ARC)
5. Arati Dangre (M.S./Ph.D.) Summer 2005
6. Shuzao Li (Ph.D. January 2007)